PATENT COOPERATION TREATY

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INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

(Chapter II of the Patent Cooperation Treaty)

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Form PCT/IPEA/416
15009pc1		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/DK2004/000494	09.07.2004	14.07.2003

International Patent Classification (IPC) or national classification and IPC
C12Q1/68

Applicant
STATENS SERUM INSTITUT

1. This report is the international preliminary examination report, established by this International Preliminary Examining Authority under Article 35 and transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 9 sheets, including this cover sheet.
3. This report is also accompanied by ANNEXES, comprising:
   a. ☐ *sent to the applicant and to the International Bureau)* a total of 2 sheets, as follows:
      ☐ sheets of the description, claims and/or drawings which have been amended and are the basis of this report and/or sheets containing rectifications authorized by this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions).
      ☐ sheets which supersede earlier sheets, but which this Authority considers contain an amendment that goes beyond the disclosure in the international application as filed, as indicated in item 4 of Box No. I and the Supplemental Box.
   b. ☐ *(sent to the International Bureau only)* a total of (indicate type and number of electronic carrier(s)) , containing a sequence listing and/or tables related thereto, in computer readable form only, as indicated in the Supplemental Box Relating to Sequence Listing (see Section 802 of the Administrative Instructions).

4. This report contains indications relating to the following items:

☒	Box No. I	Basis of the opinion
☒	Box No. II	Priority
☐	Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
☐	Box No. IV	Lack of unity of invention
☒	Box No. V	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
☐	Box No. VI	Certain documents cited
☐	Box No. VII	Certain defects in the international application
☐	Box No. VIII	Certain observations on the international application

Date of submission of the demand	Date of completion of this report
11.02.2005	06.10.2005

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INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

International application No.
PCT/DK2004/000494

Box No. I Basis of the report

1. With regard to the **language**, this report is based on the international application in the language in which it was filed, unless otherwise indicated under this item.

   ☐ This report is based on translations from the original language into the following language , which is the language of a translation furnished for the purposes of:
   - ☐ international search (under Rules 12.3 and 23.1(b))
   - ☐ publication of the international application (under Rule 12.4)
   - ☐ international preliminary examination (under Rules 55.2 and/or 55.3)

2. With regard to the **elements*** of the international application, this report is based on *(replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report)*:

Description, Pages

1-51 as originally filed

Sequence listings part of the description, Pages

1-10 received on 26.10.2004 with letter of 22.10.2004

Claims, Numbers

1-14 filed with telefax on 07.09.2005

Drawings, Sheets

1/4-4/4 as originally filed

☒ a sequence listing and/or any related table(s) - see Supplemental Box Relating to Sequence Listing

3. ☒ The amendments have resulted in the cancellation of:
   - ☐ the description, pages
   - ☒ the claims, Nos. 15-38
   - ☐ the drawings, sheets/figs
   - ☐ the sequence listing *(specify)*:
   - ☐ any table(s) related to sequence listing *(specify)*:

4. ☐ This report has been established as if (some of) the amendments annexed to this report and listed below had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).
   - ☐ the description, pages
   - ☐ the claims, Nos.
   - ☐ the drawings, sheets/figs
   - ☐ the sequence listing *(specify)*:
   - ☐ any table(s) related to sequence listing *(specify)*:

* *If item 4 applies, some or all of these sheets may be marked "superseded."*

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

International application No.
PCT/DK2004/000494

Box No. II Priority

1. ☒ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:
   - ☐ copy of the earlier application whose priority has been claimed (Rule 66.7(a)).
   - ☒ translation of the earlier application whose priority has been claimed (Rule 66.7(b)).

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid (Rule 64.1). Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:

Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-14
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-14
Industrial applicability (IA)	Yes:	Claims	1-14
	No:	Claims	

2. Citations and explanations (Rule 70.7):

see separate sheet

Supplemental Box relating to Sequence Listing

Continuation of Box I, item 2:

1. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application and necessary to the claimed invention, this report has been established on the basis of:

   a. type of material:

   ☒ a sequence listing

   ☐ table(s) related to the sequence listing

   b. format of material:

   ☒ in written format

   ☒ in computer readable form

   c. time of filing/furnishing:

   ☐ contained in the international application as filed

   ☐ filed together with the international application in computer readable form

   ☒ furnished subsequently to this Authority for the purposes of search and/or examination

   ☒ received by this Authority as an amendment on

2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table(s) relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional observations, if necessary:

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

D1: RICH CHANTAL ET AL: "Identification of human enterovirulent Escherichia coli strains by multiplex PCR" JOURNAL OF CLINICAL LABORATORY ANALYSIS, vol. 15, no. 2, 2001, pages 100-103, XP008038376 ISSN: 0887-8013

D2: JP 2003 164282 A (RAKAN:KK; GIFU UNIV) 10 June 2003 (2003-06-10)

D3: EP-A-0 556 504 (SHIMADZU CORP) 25 August 1993 (1993-08-25)

D4: WO 01/94634 A (BIOPOOL INT INC) 13 December 2001 (2001-12-13)

D5: WO 02/36827 A (AUSUBEL FREDERICK M ; GEN HOSPITAL CORP (US); KUDVA INDIRA (US); CALDE) 10 May 2002 (2002-05-10)

D6: WO 03/010332 A (SCHINKINGER MANFRED ; VOLLENHOFER-SCHRUMPF SABINE (AT); FRAENZL GERT () 6 February 2003 (2003-02-06)

D7: WO 95/29261 A (UNIV HAWAII) 2 November 1995 (1995-11-02)

D8: WO 01/48237 A (HOEFT ANDREAS ; STUEBER FRANK (DE)) 5 July 2001 (2001-07-05)

D9: WO 02/053771 A (BIOTECON) 11 July 2002 (2002-07-11)

D10: WO 99/63112 A (FRASER MARK S ; HUNT WESSON INC (US); ROMICK THOMAS L (US)) 9 December 1999 (1999-12-09)

D11: WO 92/17609 A (HOLMES MICHAEL JOHN ; DYNAL AS (NO)) 15 October 1992 (1992-10-15)

D12: WO 00/61720 A (NERENBERG MICHAEL I ; EDMAN CARL F (US); METHA PRESHANT P (US); NANOGE) 19 October 2000 (2000-10-19)

D13: DE 101 23 183 A (BECTON DICKINSON CO) 22 November 2001 (2001-11-22)

D14: WO 00/29618 A (UNIVERISTY OF VIRGINIA PATENT FOUNDATION) 25 May 2000 (2000-05-25)

D15: PATON A W ET AL: "Direct detection and characterization of shiga toxigenic Escherichia coli by multiplex PCR for stx1, stx2, eae, ehxA, and saa" JOURNAL OF CLINICAL MICROBIOLOGY 2002 UNITED STATES, vol. 40, no. 1, 2002, pages 271-274, XP002304663 ISSN: 0095-1137

D16: WO 01/46477 A (CONAGRA GROCERY PRODUCTS COMPA) 28 June 2001 (2001-06-28)

D17: TOMA C ET AL: "Multiplex PCR assay for identification of human diarrheagenic Escherichia coli" JOURNAL OF CLINICAL MICROBIOLOGY 01 JUN 2003 UNITED STATES, vol. 41, no. 6, 1 June 2003 (2003-06-01), pages 2669-2671, XP002304664 ISSN: 0095-1137

1 Claim 1

D17 is considered the closest prior art for the subject matter of claim 1 and discloses a method for simultaneous detection of diarrheagenic *E. coli* groups EPEC, ETEC, VTEC (these are the strains that comprise a verotoxin or shigatoxin gene, which are in D17 referred to as STEC), and EIEC by testing for the presence of the genes eae, vtx (called stx in D17), ipaH, sta (called est in D17), elt and aggR (Tables 1 and 2). D17 implicitly detects also Shigella via the ipaH gene. The method is based on primers chosen to match several clinical subtypes of the virulence gene. The method is performed as a multiplex PCR which comprises a PCR setup designed to enclose all primer sets in one single reaction, leading to the specific amplification of any given template present (see result in Figure 1).

1.1 Claim 1 differs from D17 in that the presence of strains with the ehxA gene is detected along with the other strains, which are a subgroup of STEC (or VTEC) according to the description.

No particular technical effect appears to be associated with this difference.

The problem solved by claim 1 can therefore be seen as the provision of an alternative target for detecting diarrheagenic *E.coli*.

D15 and D16 disclose the detection of ehxA in conjunction with the detection of other markers as a means to characterise diarrheagenic *E. coli* strains. D15 detects ehxA after a multiplex PCR reaction together with vtx1, vtx2, eae and saa in order to determine if a VTEC strain is more likely to be associated with severe disease (Abstract and Conclusions). In D16 a probe for the enterohemolysin encoding gene of *E. coli*, ehxA, is put on an array together with probes targeted at vtx2, eae and *E. coli* 23S rRNA (page 20; Figure 4). Therefore the use of ehxA as one of the targets for detecting diarrheagenic *E.coli* was already known from the prior art. The person skilled in the art who wanted to use an alternative target for the ones in the assay of D17, would have made an arbitrary selection amongst the targets in the prior art, and one of the possibilities would be to use ehxA as a target. The solution of claim 1 therefore cannot be considered inventive (Article 33(3) PCT).

1.2 Claim 1 additionally differs from D17 in that the screening method incorporates a

positive control and uses the UNG system.

The technical effect of this difference is that the robustness of the procedure is improved. The further problem solved by the subject matter of claim 1 can therefore be seen in the provision of a more robust method.

However, using a positive control for a screening method of detecting bacteria is a well known feature, e.g. in D10 (page 17) a universal 16S rRNA probe is used, in D16 a 23S rDNA probe for E. coli is incorporated. Also the use of the UNG system in a mutiplex amplification system is a standard measure to improve te robustness of the method. Hence, the inclusion of these features in order to solve the above stated problem does not require inventive activity from the person skilled in the art.

1.3 Claim 1 further differs from D17 in that it specifies the primers used for the amplification to be selected from Table 3. As only one primer has to be selected for a method to fall within the scope of claim 1, and as D17 discloses a primer for ipaH amplification which encompasses the primer with SEQ ID NO 17, the difference amounts to a modified primer for the amplification of ipaH. No technical effect appears to be associated with this difference, therefore the problem to be solved was to provide an alternative primer. The primer of Table 3 with SEQ ID 17 would be one of many alternatives from which the person skilled in the art would select one or more without inventive skill.

Other primers of Table 3 are also not considered inventive as they are variants of amplification primers disclosed in similar contexts.

D2 discloses a nucleic acid molecule according to SEQ ID NO 176 which is identical to a primer with SEQ ID No 2 of Table 3.

D3 discloses nucleic sequences of 20 bp (SEQ ID No 11 and 19) which are parts of the primers with SEQ ID Nos 1 and 7 disclosed in Table 3, D4 discloses a part of 21 nucleotides of the primer with SEQ ID No 13 of Table 3 (Primer Slt1), D5 discloses a nucleic acid comprising a part of 18 nucleotides of the primer with SEQ ID No 14 of Table 3 (SEQ ID No 20). D6 discloses a nucleic acid molecule with a sequence which comprises a 16 nucleotides part of the primer with SEQ ID No 15 of Table 3 (SEQ ID No 7), D7 disclose a nucleic acid which comprises a 19 nucleotides part of the primer with SEQ ID No 17 of Table 3 (SEQ ID No 6), D8 disclose a nucleic acid which comprises a 19 nucleotides part of the primer with SEQ ID No 24 of Table 3 (Primer sknl) and D9 discloses nucleic acids which comprise the primer with SEQ ID 16 of Table 3 (Probes with SEQ ID NO 24,25 and 39). D17 discloses a primer for ipaH amplification which encompasses the primer with SEQ ID NO 17. Hence, the documents D3-D8, 17

disclose already primers related to those of Table 3, hence the inclusion of these features in the method of claim 1 does not render the claim inventive (Article 33(3) PCT).

Claim 1 is hence not considered inventive (Article 33(3) PCT).

The application states that the inventive aspect of the method lies in its ability to distinguish 2 different types of VTEC strains from each other, with important implications for the clinical treatment of infections with these respective strains. This would be achieved by using detecting eae, and vtx1 and vtx2. However, this is not reflected in the claims in their current form, as the claims encompass many embodiments which do not detect the combination of these 3 genes, and therefore do not have the technical effect of distinguishing 2 clinically relevant subgroups of VTEC.

2 Claim 11

Several documents disclose nucleic acids which are able to prime or hybridise to the genes ipaH, elt, eae and st (e.g. in D1) or to ehxA, eae and vtx1, vtx2 (e.g. D15). To combine such nucleic acids in a kit cannot be considered inventive (Article 33(3) PCT).

3 Claim 10 and 13

The specification of the probes in claim 7 or 15 does not render the screening method inventive, as nucleic acids with such sequences were already disclosed previously.

D2 discloses a nucleic acid molecule which comprises 18 nucleotides of the probe sequence SEQ ID 27 of Table 7 (SEQ ID No 18). D4 discloses a nucleic acid molecule which comprises 17 nucleotides of the probe sequence SEQ ID 28 of Table 7 (SEQ ID No 178). D9 discloses a nucleic acid molecule which comprises 22 nucleotides of the probe sequence SEQ ID 30 of Table 7 (SEQ ID No 82).

D10 discloses a nucleic acid molecule which comprises 17 nucleotides of the probe sequence SEQ ID 26 of Table 7 (SEQ ID No 27). D11 discloses a nucleic acid molecule which comprises 18 nucleotides of the probe sequence SEQ ID 29 of Table 7 (primer 4). D12 discloses a nucleic acid molecule which comprises 22 nucleotides of the probe sequence SEQ ID 31 of Table 7 (SEQ ID No 45). D13 discloses a nucleic acid molecule which comprises 31 nucleotides which have at least 80% identity to the probe sequence SEQ ID 32 of Table 7

(SEQ ID No 36). D14 discloses a nucleic acid which is a 20 nucleotide part of the probe with SEQ ID No 36 (SEQ ID No 1). Hence, D2, D4, D9-D14 each disclose polynucleotides as in claims 10 or 13, the incorporation of these features does not render the claim to which they refer inventive.

4 Claim 9 and 12

Claims 2 and 14 refer to a primer selected from the group consisting amongst others of the primer sequences of table 3 (SEQ ID Nos 1-25).
D2 discloses a nucleic acid molecule according to SEQ ID NO 176 which is identical to a primer with SEQ ID No 2 of Table 3.

D3 discloses nucleic sequences of 20 bp (SEQ ID No 11 and 19) which are parts of the primers with SEQ ID Nos 1 and 7 disclosed in Table 3, D4 discloses a part of 21 nucleotides of the primer with SEQ ID No 13 of Table 3 (Primer Slt1), D5 discloses a nucleic acid comprising a part of 18 nucleotides of the primer with SEQ ID No 14 of Table 3 (SEQ ID No 20). D6 discloses a nucleic acid molecule with a sequence which comprises a 16 nucleotides part of the primer with SEQ ID No 15 of Table 3 (SEQ ID No 7), D7 disclose a nucleic acid which comprises a 19 nucleotides part of the primer with SEQ ID No 17 of Table 3 (SEQ ID No 6), D8 disclose a nucleic acid which comprises a 19 nucleotides part of the primer with SEQ ID No 24 of Table 3 (Primer sknI) and D9 discloses nucleic acids which comprise the primer with SEQ ID 16 of Table 3 (Probes with SEQ ID NO 24,25 and 39). Hence, the documents D3-D8 disclose already primers related to those of claims 9 and 12, hence the inclusion of these features in the method of claim 1 does not render the claim inventive (Article 33(3) PCT). The inclusion of such probes in a kit according to claim 11 is also not considered inventive as these are some of numereous alternatives from which a person skilled in the art would select without inventive skill.

Claims

1. A screening method for simultaneous detection of diarrheagenic *Shigella spp.* and *E. coli* (DEC) including A/EEC & EPEC, ETEC, VTEC, EIEC and strains with the *ehxA* gene, wherein said method:
   a) comprises detecting *Shigella spp.* by detecting the presence of the *ipaH* gene;
   b) incorporates a 16S rDNA positive control;
   c) is based on primers chosen to match all clinically relevant subtypes of the given virulence gene;
   d) is performed with multiplex PCR;
   e) comprises a PCR setup designed to enclose all primer sets in one single reaction, leading to the specific amplification of any given template present;
   f) uses primers selected from table 3;
   g) uses the UNG system

2. A screening method according to claim 1, detecting the genes selected from the group comprising: *ipaH, eae, sta, vtx1, vtx2,* and *elt,* parts of these genes or products of these genes or parts thereof, such as RNA or polypeptides.

3. A method according to claim 1, detecting the genes selected from the group comprising: *ipaH, eae, ehxA, sta, vtx1, vtx2, elt,* and *bfpA,* parts of these genes or products of these genes or parts thereof, such as RNA or polypeptides

4. A screening method according to any of the preceding claims where the genes are detected by size identification, e.g. by agarose gel electrophoresis or capillary electrophoresis.

5. A screening method according to any of the preceding claims where the genes are detected with a hybridisation probe.

6. A screening method according to claim 5, where the probes are selected from table 7.

7. A screening method according to any of the preceding claims where the material to be analysed can be any material from where bacteria can be extracted, e.g stool samples, consumables etc.

8. A screening method of any of the preceding claims, in which the testing is carried out on a sample, such as a sample from a human or an animal (ie. stool), a sample from a consumable products (ie. food and beverages), a bacteria culture or a sample from sewage.

9. A screening method according to any of the preceding claims, in which the primers used are selected from the group consisting of:
a) the primers of table 3,
b) sequences having a sequence identity of at least 80% (such as a least 85%, at least 90%, or at least 95%) with the primer sequences of a)
c) parts of the sequences in a) or b), having a lenght of more than 10, preferred more than 13 nucleotides (eg. consisting of 14, 15, 16, 17, 18, 19, 20, 21 or 22 consequtive nucleotides),
d) sequences comprising a sequence in a), b) or c), said sequence having a lenght of at least most 100 nucleotides, such as at most 90, 80, 70, 60, 50, 40, or at most 30 nucleotides.

10. A screening method according to any of the preceding claims, in which the probes used are selected from the group consisting of:
a) the probe sequences of table 7,
b) sequences having a sequence identity of at least 80% (such as a least 85%, at least 90%, or at least 95%) with the primer sequences of a)
c) parts of the sequences in a) or b), having a lenght of more than 10, preferred more than 16 nucleotides, such as more than 17, 18, 19 or 20 nucleotides (eg. consisting of 14, 15, 16, 17, 18, 19, 20, 21 or 22 consequtive nucleotides of the sequences in a) or b)),
d) sequences comprising a sequence in a), b) or c), said sequence having a lenght of at least most 100 nucleotides, such as at most 90, 80, 70, 60, 50, 40, or at most 30 nucleotides.

11. A kit which comprises, in a single or in separate containers, nucleotide sequences which are able to prime amplification in a nucleotide sequence amplification reaction, such as PCR, of the genes: *ipaH, eae, sta, vtx1, vtx2,* and *elt* or parts of these genes or the complementary strands to the genes or parts thereof and which comprises a means for a control, such as primers for 16S rDNA .

12. A kit according to claim 11, in which the nucleotide sequences for priming are selected from the group consisting of the priming sequences in table 3.

13. A kit according to claim 11, in which the nucleotide sequences for probing are selected from the group consisting of the probe sequences in table 7.

14. A kit according to any of the preceding claims, which comprises a means for detecting by size identification, ie. an agarose gel or a capillary tube optionally filled with buffer.